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(57) Abstract

Methods for engineering transgenic organisms that synthesize polyhydroxyalkanoates (PHAs) containing 3–hydroxyhexanoate as comonomer have been developed. These processes are based on genetically engineered bacteria such as *Escherichia coli* or in plant crops as production systems which include PHA biosynthetic genes from PHA producers. In a preferred embodiment of the method, additional genes are introduced in wild type or transgenic polyhydroxybutyrate (PHB) producers, thereby creating new strains that synthesize 3HH monomers which are incorporated into PHAs. The 3HH monomer preferably is derived in microbial systems using butanol or butyrate as feedstocks, which are precursors of 3–hydroxyhexanoyl–CoA. Pathways for *in vivo* production of butyrol–CoA specifically encompassing butyryl–CoA dehydrogenase activity are provided.

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TRANSGENIC SYSTEMS FOR THE MANUFACTURE OF POLY(3-HYDROXY-BUTYRATE-CO-3-HYDROXYHEXANOATE)

Background Of The Invention

The present invention is generally in the field of polyhydroxyalkanoate materials, and more particularly to improved methods of production thereof.

Polyhydroxyalkanoates (PHAs) are natural, thermoplastic polyesters and can be processed by traditional polymer techniques for use in an enormous variety of applications, including consumer packaging, disposable diaper linings and garbage bags, food and medical products. Methods which can be used for producing PHA polymers from microorganisms which naturally produce polyhydroxyalkanoates are described in U.S. Patent No. 4,910,145 to Holmes, et al.; Byrom, "Miscellaneous Biomaterials" in *Biomaterials* (Byrom, ed.) pp. 333-59 (MacMillan Publishers, London 1991); Hocking and Marchessault, "Biopolyesters" in *Chemistry and Technology of Biodegradable Polymers* (Griffin, ed.) pp. 48-96 (Chapman & Hall, London 1994); Holmes, "Biologically Produced (R)-3-hydroxyalkanoate Polymers and Copolymers" in Developm*ents in Crystalline Polymers* (Bassett, ed.) vol. 2, pp. 1-65 (Elsevier, London 1988); Lafferty *et al.*, "Microbial Production of Poly-b-hydroxybutyric acid" in *Biotechnology* (Rehm & Reed, eds.) vol. 66, pp. 135-76 (Verlagsgesellschaft, Weinheim 1988); Müller & Seebach, *Angew. Chem. Int. Ed. Engl.* 32:477-502 (1993). The natural biosynthetic pathway for production of polyhydroxybutyrate (PHB) is shown in Figure 1.

Methods for producing PHAs in natural or genetically engineered organisms are described by Steinbüchel, "Polyhydroxyalkanoic Acids" in *Biomaterials* (Byrom, ed.) pp. 123-213 (MacMillan Publishers, London 1991); Williams & Peoples, *CHEMTECH*, 26:38-44 (1996); Steinbüchel & Wiese, *Appl. Microbiol. Biotechnol.*, 37:691-97 (1992); U.S. Patent Nos. 5,245,023; 5,250,430; 5,480,794; 5,512,669; 5,534,432 to Peoples and Sinskey (which also disclose and claim the genes encoding reductase, thiolase, and PHB polymerase); Agostini *et al., Polym. Sci.*, Part A-1,

9:2775-87 (1971); Gross *et al., Macromolecules*, 21:2657-68 (1988); Dubois, *et al., Macromolecules*, 26:4407-12 (1993); Le Borgne & Spassky, *Polymer*, 30:2312-19 (1989); Tanahashi & Doi, *Macromolecules*, 24:5732-33 (1991); Hori *et al., Macromolecules*, 26:4388-90 (1993); Kemnitzer *et al., Macromolecules*, 26:1221-29 (1993); Hori *et al., Macromolecules*, 26:5533-34 (1993); Hocking & Marchessault, Polym. Bull., 30:163-70 (1993); Xie et al., Macromolecules, 30:6997-98 (1997); and U.S. Patent No. 5,563,239 to Hubbs et al. A general pathway for production of PHAs is shown in Figure 2. Synthetic polymer synthesis approaches including direct condensation and ring-opening polymerization of the corresponding lactones are described in Jesudason & Marchessault, *Macromolecules* 27:2595-602 (1994); U.S. Patent No. 5,286,842 to Kimura; U.S. Patent No. 5,563,239 to Hubbs *et al.*; U.S. Patent No. 5,516,883 to Hori *et al.*; U.S. Patent No. 5,461,139 to Gonda *et al.*; and Canadian Patent Application No. 2,006,508. WO 95/15260 describes the manufacture of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) films, and U.S. Patent Nos. 4,826,493 and 4,880,592 to Martini et al. describe the manufacture of PHB and PHBV films. U.S. Patent No. 5,292,860 to Shiotani et al. describes the manufacture of the PHA copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHH).

To date, PHAs have seen limited commercial availability, with only the copolymer PHBV being available in development quantities. This copolymer has been produced by fermentation of the bacterium *Ralstonia eutropha*. Fermentation processes for production of other PHAs have been developed (Williams & Peoples, *CHEMTECH* 26: 38-44 (1996)). Plant crops are also being genetically engineered to produce these polymers, offering a cost structure in line with the vegetable oils and direct price competitiveness with petroleum-based polymers (Williams & Peoples, *CHEMTECH* 26: 38-44 (1996)).

Several factors are critical for economic biological production of PHAs, including substrate costs, fermentation time, and efficiency of downstream processing. For large-scale fermentations of commodity

products, it is generally known that plasmid-based systems are unsatisfactory due to the extra burden of maintaining the plasmids and problems in maintaining stable expression.

Known biological systems for the production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) are inefficient. For example, Shimamura, et al., *Macromolecules*, 27:878 (1994) discloses that *Aeromonas caviae* synthesizes a PHA composed of 3-hydroxybutyrate and 3-hydroxyhexanoate (3HH) when grown on olive oil or C_{12} to C_{18} fatty acids. The fraction of the 3HH monomer was determined to be dependent on the concentration of the carbon source and the fermentation time and could amount to levels of 25% (Doi, et al., *Macromolecules*, 28: 4822 (1995)). As a result of increasing 3HH substrate levels, the crystallinity, melting temperature, and glass-transition temperature of the PHA decrease. These changes in physical properties lead to an increased susceptibility to degradation by PHB depolymerase from *Alcaligenes faecalis*. Other natural microorganism that incorporate low levels of 3HH in a PHB copolymer are *Comamonas testosteroni* and *Bacillus cereus* (Huisman, et al., *Appl. Environ. Microbiol.* 55: 1949 (1989); Caballero, et al., *Int. J. Biol. Macromol.*, 17: 86 (1995)). Recombinant *Pseudomonas putida* GPp104 strains in which the *phb* genes from either *Thiocapsia pfenigii* or *Chromatium vinosum* were introduced also accumulated PHA with 3-hydroxyhexanoate as major constituent.

PHAs generally are divided into two classes based on the polymer composition: short side-chain PHAs and long side-chain PHAs. Incorporation of monomers from one group into a PHA belonging to the other usually is limited to low levels. In some cases where the monomers are abundant for both PHAs, the bacterium generally produces separate PHA granules each comprising one type of PHA. Substrate specificities of the PHA polymerases therefore can be generalized as optimal for short side-chains (C_4 and C_5) or medium side-chains (C_8-C_{10}). Based on composition of PHAs synthesized by individual microorganisms, PHA polymerases that incorporate 3-hydroxyhexanoate can be identified. Thus, PHA polymerases

from *A. caviae*, *C. testosteroni* and *T. pfenigii* are known for incorporating 3-hydroxyhexanoate into the PHA, whereas the enzymes from *Paracoccus denitrificans, Sphaerotilus natans* and *Rhodococcus sp.* have a preference for 3-hydroxyvalerate. The PHA polymerases from the latter organisms also are useful in making PHB-co-HH copolymers, due to their preference for C_5 over C_4. Unfortunately, however, these bacteria generally have a low growth rate, often are difficult to break open, and have only a limited amenability to genetic engineering. It is thus desirable to develop efficient, more cost-effective ways of producing PHAs containing 3H-co-HH by biological systems.

It is therefore an object of the present invention to provide genetically engineered systems for the production of polyhydroxyalkanoate polymers including 3-hydroxyhexanoate monomers (HHPHA).

It is another object of this invention to provide useful mutations which can be used to produce 3-hydroxyhexanoic monomers from more economic feedstocks, such as butyrate or butanol.

It is a further object of this invention to provide genes suitable for converting cellular metabolites derived from carbohydrate feedstocks to Butyryl-CoA for the production of 3-hydroxyhexanoate comonomers.

It is another object of this invention to provide improved methods of producing PHAs containing 3-hydroxyhexanoate as comonomer.

It is still another object of this invention to provide new pathways in biological systems for the endogenous synthesis of the 3-hydroxyhexanoate monomer.

It is a further object of this invention to provide genetically engineered biological systems for production of PHAs containing 3-hydroxyhexanoate in which expression is sufficient and stable.

Summary Of The Invention

It has been discovered that biological systems for the production of PHAs containing 3-hydroxy-co-hydroxyhexanoate (3H-co-HH) can be improved by using transgenic organisms with faster growth rates and/or by

genetically engineering these organisms to produce the co-monomer 3-hydroxyhexanoic acid from cheaper feedstocks, such as butyrate or butanol, or directly from glucose by incorporating genes encoding enzymes which can channel cellular intermediates to butyryl-CoA, thereby improving the economics of PHA production using transgenic organisms. These processes are based on genetically engineered bacteria such as *Escherichia coli* or on plant crops as production systems which include PHA biosynthetic genes from PHA producers such as *R. eutropha* and *P. putida*. In a preferred embodiment of the method, additional genes are introduced in transgenic PHB producers, thereby creating new strains that synthesize monomers such as 3HH which are incorporated into PHAs.

In a preferred embodiment of the methods, microorganisms which do not normally produce the storage polymer PHAs are genetically engineered to produce PHAs by the introduction of a PHA synthase gene and additional transgenes selected from the group comprising genes encoding ß-ketothiolase, acetoacetyl-CoA reductase, ß-ketoacyl-CoA reductase, enoyl-CoA hydratase and ß-hydroxyacyl-ACP-coenzymeA transferase. The genes are preferably selected on the basis of the substrate specificity of their encoded enzymes being beneficial for the production of the 3HH polymers. Useful mutations that can be used to produce 3-hydroxyhexanoic monomers from more economic feedstocks, such as butyrate or butanol, are described. These mutants can be readily generated in bacteria suitable for practising the described invention by standard techniques known to those skilled in the art.

Methods for engineering transgenic organisms that synthesize PHAs containing 3-hydroxyhexanoate as comonomer have been developed. In a preferred embodiment of these systems, the method is used to engineer either (1) a bacterium such as *Escherichia coli, Klebsiella, Ralstonia eutropha, Alcaligenes latus, Pseudomonas putida* or other microorganisms that are able to synthesize PHAs, or (2) a higher plant, such as the seed of an oil crop (e.g., Brassica, sunflower, soybean, corn, safflower, flax, palm or coconut) or starch accumulating plants (e.g., potato, tapioca, or cassava). These are screened to identify enzyme activities desirable for conversion of metabolic

intermediates into *R*-3-hydroxyhexanoyl CoA, specifically butyryl CoA dehydrogenase activity and acyl CoA:ACP transferase activities. The latter conversion is catalyzed either by a single protein or by a combination of thioesterase and acyl CoA synthase activities. The flux of normal cellular metabolites to 3-hydroxyhexanoate is redirected via one or more of three different pathways. These three pathways generate 3-hydroxyhexanoate, either (1) using a butyrate fermentation pathway from *Clostridium acetobutylicium*, (2) using fatty acid biosynthetic enzymes from *E. coli*, or (3) using the fatty acid oxidation complex from *Pseudomonas putida*. Examples demonstrate a bacterium expressing a functional PHA synthase from a transgene is described, along with methods for expressing these genes in transgenic plant crops.

Methods to select genes that encode enzymes which convert crotonyl CoA to butyryl CoA are provided, as well as screening methods that identify enzymes that convert acyl ACP intermediates into acyl CoA or into acyl CoA precursors for PHA biosynthesis. Transgenic *E. coli* strains in which a gene encoding a PHA polymerase is integrated in the chromosome and expressed to levels supporting PHA synthesis are provided. Such transgenic strains, which also have specific mutations on the chromosome, allow the selection and screening of these activities using genomic libraries from different biological sources.

Procedures are described for engineering new pathways in biological systems for the endogenous synthesis of the 3-hydroxyhexanoate monomer. In a preferred embodiment, *E. coli* is engineered to synthesize PHBH from either inexpensive carbohydrate feedstocks such as glucose, sucrose, xylose and lactose or mixtures of such carbohydrates and fatty acids as the only carbon source by introducing genes encoding enzymes that convert cellular metabolites to 3-hydroxyhexanoyl CoA into the *E. coli*. For efficient PHA synthesis in recombinant *E. coli* strains, it is crucial that the expression of all the genes involved in the pathway be adequate. To this end, the genes of interest can be expressed from extrachromosomal DNA molecules such as plasmids, which intrinsically results in a copy-number effect and

consequently high expression levels, or they can be expressed from the chromosome. For large-scale fermentations of commodity products it is generally known that plasmid-based systems are unsatisfactory due to the extra burden of maintaining the plasmids and the problems of stable expression. These drawbacks can be overcome using chromosomally encoded enzymes and/or by improving the transcriptional and translational signals preceding the gene of interest such that expression is sufficient and stable.

Brief Description of the Drawings

Figure 1 is a schematic of a pathway for biosynthesis of PHB.

Figure 2 is a schematic of a general pathway for biosynthesis of PHA.

Figure 3 is a schematic of a preferred pathway for biosynthesis of PHBH using the *Clostridium acetbutylicum* butyrate fermentation pathway.

Figure 4 is a schematic of a preferred pathway for biosynthesis of PHBH using the fatty acid oxidation pathway.

Figure 5 is a schematic of a preferred pathway for biosynthesis of PHBH using the fatty acid pathway.

Figure 6 is a schematic of construction of pMLXp11C7cat and pMLXp13C7cat for integration of the PHA polymerase gene from *N. salmonicolor* on the chromosome of *E. coli*.

Figure 7 is a schematic of selection for crotonase and hydroxybutyryl CoA dehydrogenase genes by complementation of an *E. coli. fadB* mutation.

Figure 8 is a schematic of selection for butyryl CoA dehydrogenase genes by complementation of an *E. coli.* strain that is phenotypically *fadE* defective.

Figure 9 is a schematic of selection for a PHBH recombinant pathway in *E. coli.* using the PHA polymerase gene *phaC* from *P. Putida*.

Figure 10 is a schematic of a preferred screening procedure for genes encoding enzymes that convert acyl ACP to acyl CoA with the use of the *Vibrio fischeri lux* system.

Detailed Description Of The Invention

Metabolism of any HA production organism, including bacteria and plant crops, can be redirected to supply specific metabolites for PHA synthesis by metabolic engineering. In order to make this approach effective, it is necessary to develop new biochemical pathways leading to the desired monomer from common metabolic intermediates. It is not necessary that such pathways exist in one organism as the individual steps can be reconstituted in the production organism of choice using genetic engineering techniques. Processes developed to incorporate alternative monomers that are derived from supplemented feedstocks have specific drawbacks. First, adding supplemental feeds into a fermenter are costly as they expand the infrastructure and impose additional quality control. Second, addition of monomer precursors in the feed needs to be tightly controlled to achieve a constant composition of the monomer pools and PHA composition.

A similar approach in metabolic engineering methods have therefore been developed which allow production of PHBH in organisms, such as *R. eutropha, C. Testosteroni, A. latus, A. vinelandii* and *P. denitrificans*, as well as in transgenic microbial and plant crop systems expressing a PHA synthase from a heterologous gene or genes.

I. Polyhydroxyalkanoates

Several types of PHAs are known. It is useful to broadly divide the PHAs into two groups according to the length of their side chains and according to their pathways for biosynthesis. Those with short side chains, such as polyhydroxybutyrate (PHB), a homopolymer of R-3-hydroxybutyric acid units, are crystalline thermoplastics; PHAs with long side chains are more elastomeric. The former polymers have been known for about seventy years (Lemoigne & Roukhelman 1925), while the latter polymers are a relatively recent discovery (deSmet, *et al.*, *J. Bacteriol.*, 154:870-78 (1983)). Before this designation, however, PHAs of microbial origin containing both R-3-hydroxybutyric acid units and longer side chain units from C5 to C16 were identified (Wallen & Rowheder, *Environ. Sci. Technol.*, 8:576-79 (1974)). A number of bacteria which produce copolymers of D-3-

hydroxybutyric acid and one or more long side chain hydroxyacid units containing from five to sixteen carbon atoms have been identified more recently (Steinbuchel & Wiese, *Appl. Microbiol. Biotechnol.*, 37:691-97 (1992); Valentin *et al.*, *Appl. Microbiol. Biotechnol.*, 36: 507-14 (1992); Valentin *et al.*, *Appl. Microbiol. Biotechnol.*, 40:710-16 (1994); Abe *et al.*, *Int. J. Biol. Macromol.*, 16:115-19 (1994); Lee *et al.*, *Appl. Microbiol. Biotechnol.*, 42:901-09 (1995); Kato *et al.*, *Appl. Microbiol. Biotechnol.*, 45:363-70 (1996); Valentin *et al.*, *Appl. Microbiol. Biotechnol.*, 46:261-67 (1996); U.S. Patent No. 4,876,331 to Doi). Useful examples of specific two-component copolymers include PHB-co-3-hydroxyhexanoate (Brandl *et al.*, *Int. J. Biol. Macromol.*, 11:49-55 (1989); Amos & McInerey, *Arch. Microbiol.*, 155:103-06 (1991); U.S. Patent No. 5,292,860 to Shiotani *et al.*). Other representative PHAs are described in Steinbüchel & Valentin, *FEMS Microbiol. Lett.*, 128:219-28 (1995). Chemical synthetic methods have also been applied to prepare racemic PHB copolymers of this type for applications testing (PCT WO 95/20614, PCT WO 95/20615, and PCT WO 96/20621).

Useful molecular weights of the polymers are between about 10,000 and 4 million Daltons, and preferably between about 50,000 and 1.5 million Daltons. The PHAs preferably contain one or more units of the following formula:

$$-OCR^1R^2(CR^3R^4)_nCO-$$

wherein n is 0 or an integer; and

wherein R^1, R^2, R^3, and R^4 are independently selected from saturated and unsaturated hydrocarbon radicals, halo- and hydroxy- substituted radicals, hydroxy radicals, halogen radicals, nitrogen-substituted radicals, oxygen-substituted radicals, and hydrogen atoms.

Monomeric units generally include hydroxybutyrate, hydroxyvalerate, hydroxyhexanoate, hydroxyheptanoate, hydroxyoctanoate, hydroxynonanoate, hydroxydecanoate, hydroxyundecanoate, and hydroxydodecanoate units. PHAs can include monomers and polymers and derivatives of 3-hydroxyacids, 4-hydroxyacids and 5-hydroxyacids.

II. Methods of Preparing Polyhydroxyalkanoates

The PHAs can be prepared from a biological source such as a microorganism which naturally produces PHAs and which can be induced to produce the desired PHAs by manipulation of culture conditions and feedstocks, these or other microorganisms genetically engineered as described herein, or higher organisms, such as plants, which have been genetically engineered to produce PHAs.

Substrate Specificity of the Enzymes Required for PHA Synthesis

Suitable sources of PHA synthase genes are readily identified by analyzing the compositions of PHAs produce when grown on fatty acids and then isolating the PHA synthase genes by methods well known to those skilled in the art. Useful PHA synthase genes have been isolated from, for example, *Aeromonas caviae* (Fukui & Doi, *J. Bacteriol.* 179: 4821-30 (1997)), *Rhodospirillum rubrum* (U.S. Patent No. 5,849,894), *Rhodococcus ruber* (Pieper & Steinbuechel, *FEMS Microbiol.Lett.* 96(1): 73-80 (1992)), and *Nocardia corallina* (Hall et. al., *Can. J. Microbiol.* 44: 687-91 (1998)).

In vitro studies on PHB polymerases have shown that the enzyme from *Z. ramigera* I-16-M is strictly specific for the *R*-isomer of 3-hydroxybutyryl CoA (Fukui, et al., *Arch. Microbiol.*, 110: 149 (1976)). The PHB polymerase from *R. eutropha* is highly specific for the 3-hydroxybutyryl CoA monomer and shows only 7.5% activity towards 3-hydroxyvaleryl CoA. No activity with 3-hydroxyhexanoyl CoA or longer 3-hydroxyacyl CoA's was detected in *in vitro* studies (Haywood, et al., *FEMS Microbiol. Lett.*, 57:1 (1989)).

The NADPH-linked acetoacetyl CoA reductase from *Z. ramigera* is most active with acetoacetyl CoA, whereas 3-ketovaleryl CoA (41% of the maximal activity) and 3-ketohexanoyl CoA (0.6%) were also substrates for the enzyme (Ploux, et al., *Eur. J. Biochem.*, 174: 177 (1988)). In *R. eutropha,* the reductase activities for 3-ketovaleryl CoA and 3-ketohexanoyl CoA are respectively 48% and 3.6% of the activity that was determined for acetoacetyl CoA (Haywood, et al., *FEMS Microbiol. Lett.*, 52:259 (1988)).

In addition, *R. eutropha* has an NADH-dependent activity towards *S*-3-hydroxyacyl CoA's which is highest for the C_4 and C_8 substrates.

R.. eutropha also has two 3-ketothiolases (A and B) which have the highest activity towards the acetoacetyl CoA substrates and only 3% of the maximal activity towards 3-ketovaleryl CoA (Haywood, et al., *FEMS Microbiol. Lett.*, 52:91 (1988)). While enzyme A is 10 times more active and strictly specific for these two substrates, enzyme B also has 1-2% activity for the higher 3-ketoacyl CoA's.

In summary, the synthesis of 3-hydroxyhexanoyl-CoA monomers with the PHB enzymes from *R. eutropha* or *Z. ramigera* can be improved by identifying and using thiolase and/or reductase genes with advantageous substrate specificity for 3-ketohexanoyl-CoA. It is therefore necessary to identify and isolate genes encoding activities that can supply 3-hydroxyhexanoyl CoA for PHA biosynthesis.

Identification and isolation of phb *genes from* Nocardia salmonicolor.

N. salmonicolor is a member of the genus *Rhodococcus* which is known to incorporate high levels of 3-hydroxyvalerate into PHAs when grown on simple sugars as carbon source. This characteristic suggests that the PHB biosynthetic enzymes from *N. salmonicolor* are likely to have a wider substrate range than other PHB biosynthetic enzymes, such as those from *R. eutropha*. The genes encoding PHB polymerase and acetoacetyl CoA reductase were amplified by polymerase chain reaction using primers that were based on the nucleotide sequence of the *phaC* gene from *Rhodococcus ruber* and conserved regions in the N- and C-terminal ends of known acetoacetyl CoA dehydrogenases. DNA fragments containing the *phbB* and *phbC* genes from *N. salmonicolor* were identified in genomic digests by Southern blotting using the corresponding PCR products as probes. A 3.6 kb BamHI (*phbC*) and 4.2 kb PvuII (*phbB*) fragment were cloned into pUC119 and identified by colony blotting using the corresponding PCR products as probes.

Endogenous Formation of R-3-Hydroxyhexanoyl CoA Using the Butyrate Fermentation Pathway from Clostridium acetobutylicum.

A biosynthetic pathway that results in *R*-3-hydroxyhexanoyl CoA formation involves the elongation of butyryl CoA to 3-ketohexanoyl CoA which can subsequently be reduced to the monomer precursor, as shown in Figure 4. Butyryl CoA is formed by butyrate fermenting organisms such as *C. acetobutylicum* in a four step pathway from acetyl CoA. Elongation of butyryl CoA to 3-ketohexanoyl CoA is catalyzed by a thiolase. The complete pathway thus involves (1) the PHB biosynthetic thiolase, (2) the three enzymes from *C. acetobutylicum* that form butyryl CoA, (3) a second thiolase, specific for 3-ketohexanoyl CoA, (4) a reductase specific for this substrate, and (5) a PHB polymerase that accepts both 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

The *C. acetobutylicum* locus involved in butyrate fermentation encodes 5 enzymes/proteins: crotonase (*crt*), butyryl CoA dehydrogenase (*bcd*), 2 ETF proteins for electron transport (*etfA* and *etfB*), and 3-hydroxybutyryl CoA dehydrogenase (*hbd*) (Boynton et al., *J. Bacteriol.* 178:3015 (1996)). Another microorganism from which these genes have been isolated is *Thermoanaerobacterium thermosaccharolyticum* (van Rinsum, GenBank Acc. no.). *Hbd* and *crt* have been isolated from *C. difficile* as well (Mullany et al., *FEMS Microbiol.* Lett. 124:61 (1994)). 3-hydroxybutyryl CoA dehydrogenase activity has been detected in *Dastricha ruminatium* (Yarlett et al., *Biochem. J.* 228:187 (1995)), *Butyrivibrio fibrisolvens* (Miller & Jenesel, *J. Bacteriol.*, 138:99 (1979)), *Treponema phagedemes* (George & Smibert, *J. Bacteriol.*, 152:1049 (1982)), *Acidaminococcus fermentans* (Hartel & Buckel, *Arch. Microbiol.*, 166:350 (1996)), *Clostridium kluyveri* (Madan et al., *Eur. J. Biochem.*, 32:51 (1973)), *Syntrophospora bryanti* (Dong & Stams, *Antonie van Leeuwenhoek*, 67:345 (1995)); crotonase activity has been detected in *Butyrivibrio fibrisolvens* (Miller & Jenesel, *J. Bacteriol.*, 138:99 (1979)); and butyryl CoA dehydrogenase activity has been detected in *Megasphaera elsdenii* (Williamson & Engel, *Biochem. J.*, 218:521 (1984)), *Peptostreptococcus*

elsdenii (Engel & Massay, *Biochem. J.*, 1971, 125:879), *Syntrophospora bryanti* (Dong & Stams, *Antonie van Leeuwenhoek*, 67:345 (1995)), and *Treponema phagedemes* (George & Smibert, *J. Bacteriol.*, 152:1049 (1982)).

For all CoA-involving thiolases known so far the reaction primarily proceeds in the catabolic direction. Also, the thiolase encoded by *phbA* preferably degrades acetoacetyl CoA. Thus, in a biosynthetic pathway to 3-ketohexanoyl CoA a catabolic thiolase can be used if the reaction is being pulled in the anabolic direction by a reductase and PHA polymerase. Besides the known PHB thiolases, genes encoding these enzymes can be obtained from a range of bacteria, mammals and plants. In fact, *E. coli* has five thiolases that have been characterized poorly, both biochemically and physiologically. Two of these thiolases are encoded by previously identified genes, *fadA* and *atoB*, whereas three others are encoded by open reading frames that have not been studied. These thiolases were overexpressed and assayed with different substrates *in vitro* assays. Reductase and polymerase genes are taken from *N. salmonicolor* or any other PHA producer that incorporates C_6 monomers.

Endogenous Formation of R-3-hydroxyhexanoyl CoA Via the Fatty Acid Oxidation Pathway.

In *P. putida* monomers for PHA biosynthesis are derived from the fatty acid oxidation pathway when alkanes or oxidized alkanes are provided as carbon and energy source. The intermediate in this pathway that is channeled to PHA biosynthesis is postulated to be *S*-3-hydroxyacyl CoA (preferentially C_8 and C_{10}) which undergoes epimerization by the FaoAB complex to the *R*-isomer. The combined action of epimerase and PHA polymerase provides C_6 to C_{14} monomers for PHA. Consequently, a combination of this epimerase and a 3-hydroxyhexanoyl CoA accepting PHA polymerase provides the biosynthetic capability to synthesize PHBH from fatty acids in transgenic organisms, as shown by Figure 5. Mixtures of fatty acids and carbohydrates that are useful feedstocks for fermentative production as the 3HB monomer can be derived from acetyl CoA, whereas the 3HH component is from fatty acids. For plant crops, synthesis of the 3-

hydroxyhexanoate monomer proceeds anabolically from acetyl-CoA, or catabolically from fatty acids.

Epimerase activity has been detected in the fatty acid oxidation complexes from *E. coli* (FadAB) (Pramanik et al., *J. Bateriol.* 137:469 (1979)) and *P. fragi* FaoAB (Imamura et al., *J. Biochem.* 107: 184 (1990)). The FaoAB complex from *P. putida* KT2442 was examined after the subunits were cloned in the overexpression vector pTrcN and this complex demonstrated epimerase activity towards 3-hydroxyoctanoyl CoA, limited activity towards 3-hydroxybutyryl CoA and hardly detectable levels towards 3-hydroxyoctanoyl CoA. These results suggest that the FaoAB complex may be a determining factor in the substrate specificity of the PHA pathway in *P. putida.* Consequently, FaoAB complexes from other sources can be used to generate novel 3-hydroxyacyl CoA pools in recombinant organisms, prokaryotic, eukaryotic or archaeic. Homologous genes are readily isolated from bacteria such a *R. eutropha, A. latus, C. testosteroni, P. denitrificans, R. ruber* and other PHA and non-PHA producers using the same methods to identify the *faoAB* genes in *P. putida* KT2442.

Endogenous formation of R-3-hydroxyoctanoyl CoA via the fatty acid biosynthetic pathway.

P. putida and *P. aeruginosa* synthesize PHAs composed of medium-chain length 3-hydroxy fatty acids when grown on sugars. The predominant monomer in these PHAs is 3-hydoxydecanoate. A similar pathway can be engineered for the synthesis of PHBH in either recombinant microorganisms such as *E. coli, R. Eutropha* and *P. putida,* as well as transgenic oilseed crops, as shown by Figure 6. Besides a polymerase that accepts the 3-hydoxybutyryl CoA and 3-hydroxyhexanoyl CoA precursors, an enzymatic activity that converts 3-hydroxyacyl ACP into 3-hydroxyacyl CoA or 3-ketoacyl ACP into 3-ketoacyl CoA is required as well. Since this activity is present in *P. putida* the corresponding gene can be identified and isolated by screening procedures. Deregulation of fatty acid biosynthesis and increased activity of this pathway subsequently provides the substrate for PHBH formation.

The critical enzymatic activity in this pathway is the conversion of the 3-hydroxyacyl ACP to the CoA derivative. Thioesterases and acyl CoA synthases are widely known in their combined action can accomplish this step. Alternatively, a new activity, acyl ACP:CoA transferase, may facilitate this step in the PHA pathway and can consequently be identified in bacteria that produce PHA from oxidized carbon sources such as carbohydrates.

Growth Characteristics

For efficient PHA production, it is important that strains not lose the capability to synthesize the biopolymer for the duration of the inoculum train and the production run. Loss of any of the *phb* genes results in loss of product whereas loss of any of the genes that provide new monomers results in heterogeneous product formation. Both are undesirable and stable propagation of the strain is therefore required. Integration of the genes in the strains described in the examples was determined to be stable for at least 50 generations, sufficient for production in 250,000 L industrial fermentation vessels.

Growth and morphology of these recombinant PHA producers is not compromised by the presence of *phb* genes on the chromosome. During the selection procedures, individual integrants are selected on minimal medium plates circumventing the isolation of auxotrophic strains. Growth rates of the different *phb* integrants were similar to that of the wild-type *E. coli* strains from which the PHB producers were derived. The addition of the *phb* genes to the *E. coli* chromosome did not affect the downstream processing of these strains, as they were still easily lysed by conventional methods.

III. Applications for the Compositions

PHAs can be used in molding applications, in particular for consumer packaging items such as bottles, cosmetic containers, diaper sheets, pens, golf tees, and personal items, such as U.S. Patent Nos. 3,072,538; 3,107,172; and 4,900,299, which describe molded tampon applicators, and in films of pure PHA or blends or laminates of PHA with other materials such as starch esters or synthetic polymers. In many applications, the polymers are first

formed into fibers and the fibers are then used to construct materials such as non-woven fabrics.

The polymers can also be used in hot-melt adhesives and pressure-sensitive adhesive formulations, and to replace petrochemical polymers used in toner and developer compositions (U.S. Pat. No. 5,004,664) and as ion-conducting polymer electrolytes (U.S. Pat. No. 5,266,422). A number of features of the polyhydroxyalkanoate polymers make them particularly attractive as binders for metal powder, ceramic powder or metal/ceramic powder processing.

One of the unique features of the PHAs is that they can exist in two distinct physical forms, either as amorphous granules or as crystalline solids. PHAs therefore can be used to form a latex. PCT WO 91/13207 describes PHA latex compositions for coating paper. GB 2 292 648 A describes the use of PHA latex in architectural coating formulations. PCT WO 96/00263 describes the use of PHA latex as food coatings, in particular cheese coatings. PCT WO 92/09211 and U.S. Pat. No. 5,229,158 describe the use of PHA granule compositions for use as dairy cream substitutes. PCT WO 92/09210 and U.S. Pat. No. 5,225,227 describe the use of PHAs as flavor delivery agents in foods.

As the PHAs have become increasingly available, they have also been examined for their suitability in applications where they serve as a processing aid. One example is the use of PHA latex in the production of CRT tube components as described in PCT WO 96/17369. Key features of the usefulness of the PHAs in this application are that the coating system does not use organic solvents and that it can be readily removed during the subsequent oven treatment using less energy than conventional systems.

The PHAs can be produced in a wide variety of types depending on the hydroxyacid monomer composition (Steinbüchel & Valentin, *FEMS Microbiol. Lett.* 128: 219-28 (1995)). This wide range of polymer compositions reflects an equally wide range of polymer physical properties, including a range of melting temperatures from 40 to 180 °C, glass transition temperatures from –35 to 5 °C, degrees of crystallinity from 0% of 80%

coupled with the ability to control the rate of crystallization, and elongation to break from 5 to 500%. Poly(3-hydroxybutyrate), for example, has characteristics similar to those of polypropylene, while poly(3-hydroxyoctanoate) (a copolymer of (*R*)-3-hydroxyoctanoate and (*R*)-3-hydroxyhexanoate) types behave more like elastomers, and PHAs with longer side chains behave more like waxes. The PHAs can also be plasticized and blended with other polymers or agents.

This wide range of polymer compositions reflects an equally wide range of polymer physical properties, including solubility in organic solvents, which provides a choice of a wide range of solvents. For example, copolymers of (*R*)-3-hydroxybutyrate and other hydroxyacid comonomers have significantly different solubility characteristics from those of the PHB homopolymer. Acetone, for example, is not a good solvent for PHB, but is very useful for dissolving (*R*)-3-hydroxybutyrate copolymers with (*R*)-3-hydroxyacids containing from 6 to 12 carbon atoms (Abe, et al., *Int. J. Biol. Macromol.* 16: 115-19 (1994); Kato, et al., *Appl. Microbiol. Biotechnol.* 45: 363-70 (1996)). Similarly, Mitomo et al., *Reports on Progress in Polymer Physics in Japan*, 37: 128-29 (1994) describes the solubility of copolyesters poly(3-hydroxybutyrate-co-4-hydroxybutyrate) containing from 15 to 75 mol % 4-hydroxybutyrate residues in acetone. A number of additional solvents which are suitable for a range of PHAs have been described, for example in U.S. Patent No. 5,213,976; U.S. Patent No. 4,968,611; JP 95,135,985; JP 95,79,788; PCT WO 93/23554; DE 19533459; PCT WO 97/08931; and Brazil Pedido PI BR 93 02,312.

The compositions and methods of preparation and use thereof described herein are further described by the following non-limiting examples.

Material and Methods Used in Examples

DNA manipulations were performed on plasmid and chromosomal DNA purified with the Qiagen plasmid preparation or Qiagen chromosomal DNA preparation kits according to manufacturers' recommendations. DNA was digested using restriction enzymes (New England Biolabs, Beverly,

MA) according to manufacturers' recommendations. DNA fragments were isolated from 0.7% agarose-Tris/acetate/EDTA gels using a Qiagen kit. Oligonucleotides were purchased from Biosynthesis or Genesys. DNA sequences were determined by automated sequencing using a Perkin-Elmer ABI 373A sequencing machine. DNA was amplified using the polymerase-chain-reaction in 50 microliter volume using PCR-mix from Gibco-BRL (Gaithersburg, Md) and an Ericomp DNA amplifying machine.
Growth media and standard cloning procedures were as described by Sambrook et. al., (1992, in Molecular Cloning, a laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

PHA analysed by gas chromatographic (GC) analysis, carried out on the purified polymer or lyophilized cell mass. About 20 mg of sample was subjected to simultaneous extraction and butanolysis at 110 °C for 3 hours in 2 mL of a mixture containing (by volume) 90% 1-butanol and 10% concentrated hydrochloric acid, with 2 mg/mL benzoic acid added as an internal standard. The water-soluble components of the resulting mixture were removed by extraction with 3 mL water. The organic phase (1 μL at a split ratio of 1:50 at an overall flow rate of 2 mL/min.) was analyzed on an HP 5890 GC with FID detector (Hewlett-Packard Co, Palo Alto, CA) using an SPB-1 fused silica capillary GC column (30 m; 0.32 mm ID; 0.25 μm film; Supelco; Bellefonte, Pa.) with the following temperature profile: 80 °C, 2 min; 10 °C per min. to 250 °C; 250 °C, 2 min. Butylbenzoate was used as an internal standard.

Example 1: Isolation of Genes from *N. salmonicolor* Suitable for Improving the Production of PHBH

Transgenic *E. coli* strains that express a chromosomally encoded PHA polymerase from *N. salmonicolor* were constructed. The PHB polymerase gene from *N. salmonicolor* was isolated and a fusion of this gene was generated with the translational sequences of the PHA polymerase gene from *Z. ramigera*, which includes the N-terminal 10 residues of the *Pseudomonas* enzyme. A promoterless chloramphenicol transferase gene was then placed behind the hybrid *phbC* gene to make a *phbC-cat* fusion.

This fusion was randomly inserted into the *E. coli* chromosome using the pLOF or pUT system (Herrero et al.) and clones expressing the fusion were selected on chloramphenicol-containing growth medium. Expression of the fusion was consequently increased by selecting derivatives that are resistant to higher chloramphenicol levels.

PhaC was amplified from *N. salmonicolor* chromosomal DNA in the following reaction mix: 45 ml PCR Supermix (Gibco BRL, Gaithersburg, MD), 20 pmol of primers RSCP1 (SEQ ID NO:1)

(5' GATGCCGGTCGACCCGCGGGACCGCCGCTT CTCC)

and RSPC2 (SEQ ID NO:2)

(5' TCAGCTGAAGACGTACGTACCCGGAGC),

in 50 ml final volume for 30 cycles: 60 seconds 95 °C, 60 seconds at 55 °C and 210 seconds at 72 °C, followed by a product extension step (7 minutes at 68 °C). The *N. salmonicolor* reductase gene was amplified in the following reaction: 45 ml PCR Supermix (Gibco BRL, Gaithersburg, MD), 1 mM primers RD-up (SEQ ID NO:3)

(5' CGIGTIGCICTIGTIA CIGG)

and RD-dwn (SEQ ID NO:4)

(5' CCCATGTACAGICCICCGTT),

50 ml final volume for 30 cycles: 60 seconds 95 °C, 60 seconds at 60 °C and 210 seconds at 72 °C, followed by a product extension step (7 minutes at 68 °C). PCR products were gel purified and cloned into pCR2.1 (Invitrogen, CA). Purified fragments encoding polymerase and reductase were subsequently used in Southern blot experiments to identify a 3.6 kb *phaC* fragment and 4.6 kb *BamHI* and 4.2 kb *PvuII* fragments harboring *phaB*. Chromosomal fragments of the corresponding size were gel purified, cloned in pUC19, and clones containing the desired insert were identified by colony blot hybridization using purified *phbC* and *phbB* genes as probes.

Since efficient synthesis of PHBH requires adequate expression of genes encoding enzymes involved in the biosynthetic pathway, the *phaC* gene from *N. salmonicolor* was reconstructed to engineer strong translational

signals at the 5' end of the gene, as shown in Figure 6. *PhaC* was amplified using primers C7-5' (SEQ ID NO:5)

(5' AAGTCGACCATGCATCCGATCGGCTGGGGT)

and C7-3' (SEQ ID NO:6)

(5' ACGCTGTTCAGATCTTCGCAAGATGAATGCTAACG)

in a thermal cycling program entailing 30 seconds at 95 °C, 30 seconds at 60 °C, and 2 minutes at 72 °C, followed by a 7 minute extension at 72 °C. The reaction mix contained 47 ml PCR supermix (Gibco-BRL), 0.1 nmol of each primer, and approximately 0.05 mg of pCR2.1-*phaC7* as template. The PCR product was purified by phenol extraction and digested with *NsiI* and *BglII*. The restricted fragment then was cloned in the *PstI* and *BamHI* sites of pMSXC5cat. pMSXC5cat contains a transcriptional fusion of the PHA polymerase gene from *Z. ramigera (phaC5)* and the chloroamphenicol resistance gene. The resulting plasmid contains a translational fusion resulting in a hybrid polymerase of the N-terminal 10 amino acids from PhaC5 with the *N. salmonicolor* PHA polymerase. The resulting plasmid pMSXC7cat subsequently was digested with *AccI, HindIII*, and *FspI*, after which the *phaC* fragment was isolated for cloning behind the p_{11} and p_{13} promoters in *FseI/EcoRI* digested pMSXp_{11}AB5kan and *SmaI/EcoRI* digested pMSXp_{13}AB5kan. Fragments containing p_{11}C7cat and p_{13}C7cat were isolated as *AvrII* fragments, inserted into the *SfiI* site of pLOFHg, and integrated into the chromosome of *E. coli* MBX427, to yield pMLXp11C7cat and pMLXp13C7cat.

Alternative 3-hydroxyhexanoyl CoA accepting PHA polymerase genes can be obtained from organisms that have been shown to incorporate this monomer, including *A. caviae, C. testosteroni, T. pfenigii,* and possibly *P. denitrificans* and *S. natans*. These genes can be expressed in *E. coli* according to the same procedures described above.

Example 2: PHBH Synthesis in *E. coli* From Butyrate

Endogenous synthesis of *R*-3-hydroxyhexanoyl CoA can proceed after condensation of butyryl CoA with acetyl CoA followed by a reductive

step. This pathway requires only a broad substrate range reductase and a polymerase that accepts 3-hydroxyhexanoyl CoA. Butyrate is taken up by *E. coli* and converted to butyryl CoA by the *atoDA* gene products. Degradation of butyryl CoA is dependent on *atoB* and the *fad* regulon which is not induced by butyrate.

Plasmid pMBXc12J12 was constructed by inserting the 2.4 Kb ApoI fragment containing the *A. caviae* PHB polymerase gene (Fukui & Doi, *J. Bacteriol.* 179: 4821-30 (1997)) into the EcoRI site of pUC18. Plasmid pSU18-AB1 contains the *R. eutropha phb*AB genes under the control of an IPTG-inducible promoter in the vector pSU18 (Martinez et. al., *Gene* 66: 1659-20 (1988)). PHBH was produced from glucose and butyrate in *E. coli* MBX1325 (identical to strain DC679, *mel, fadR, atoC (con) adh*C81 (Clark & Rod, *J. Mol. Biol. Evol.* 25: 151 (1987)) containing plasmids pMBXC12J12 and pSU18-AB1 as follows. The transformed cells (1L) were grown in LB containing 20 mM butyrate for 24 hours at 30 °C and harvested by centrifugation. The PHA polymer was purified from lyophilized cells by extraction with chloroform for 16 hours and the PHA precipitated in a 5- to 10-fold excess of methanol. The precipitated polymer was analyzed by gas chromatography and identified as PHBH copolymer containing 1.0 %HH comonomer.

Example 3: PHBH Synthesis in *E. coli*
Using the Butyrate Fermentation Pathway

The butyrate fermentation pathway is shown in Figure 3. Enzymes required for 3-hydroxyhexanoate synthesis are encoded by $phbA_x$, *hbd, crt, bdh,* $phbA_y$, *phbB* and *phbC,* in which x and y indicate identical or different thiolases. The sources for these genes are *Z. ramigera* ($phbA_{xy}$), *C. acetobutylicum* (*hbd, crt, bdh*) and *N. salmonicolor* (*phbB* and *phbC*).

Crt and *hbd* were isolated by polymerase chain reaction using pC10 (Boynton et al) as template using the following primers:
5' crt (SEQ ID NO:7):

5' GGGGATCCGAATTCAGGAGGTTTTTATGGAACTAAACAA TGTCATCC;

3' crt (SEQ ID NO:8):

5' GGAATTCGAGCTCCTATCTATTTTTGAAGCC;

5' hbd (SEQ ID NO:9):

5' GGAATTCGGTACCAGGAGGTTTTTATGAAAAAGGTATGT GTTATAGG;

3' hbd (SEQ ID NO:10):

5' GGAATTCCCCGGGTTATTTTGAATAATCGTAGAAACC.

PCR products were purified, digested with *EcoRI/SacI* (crt) or *KpnI/SmaI* (hbd), and subsequently cloned in the corresponding sites of pUC18-Sfi, resulting in pMSXcrt-hbd.

Bdh was isolated by polymerase chain reaction using pC10 (Boynton et al.) as template and the following primers:

5' bcd (SEQ ID NO:11):

GGAATTCCTGCAGAGGAGGTTTTATGGATTTTAATTTAACA AGAG;

3' bcd (SEQ ID NO:12):

GGAATTCGCATGCT TATCTAAAAATTTTTCCTG.

The PCR product was purified, digested with *PstI/SphI,* and subsequently cloned in the corresponding sites of pMSXcrt-hbd, resulting in pMSXcrt-hbd-bcd.

The original operon from pC10 contained *etfAB* encoding for a putative electron transfer chain. The crt-hbd-bcd operon may not be active in the absence of this operon so the *etfA* and *etfB* genes were amplified from the pC10 (Boynton et al.) with the primers

5' etfBA (SEQ ID NO:13):

5' GGAATTCGGATCCAGGAGGTTTTATGAATATAGTTGT TTGTTTAAACA AGTTCC and

3' etfBA (SEQ ID NO:14):

5' GGAATTCGTCGACTTAATTATTAGCAGCTTTAACT TGAGC. The PCR product was purified, digested with BamHI and SalI, and

subsequently cloned in the corresponding sites of pUC18-SfiI, resulting in pMSXetfAB. The *etfAB* genes can be easily cloned into the pMSXcrt-hbd-bcd plasmid in the BamHI/SalI sites.

Example 4: PHBH Synthesis in *E. coli* Using a Fatty Acid Oxidation Pathway

The fatty acid oxidation pathway is shown in Figure 4. R-3 hydroxyhexanoyl CoA can be obtained from fatty acid oxidation intermediates by epimerization of *S*-3-hydroxyhexanoyl-CoA, reduction of 3-ketohexanoyl-CoA or by hydration of the enoyl-CoA by D-specific hydratase. The *E. coli* strain MBX240 is a derivative of the strain XL1-Blue (Stratagebe, San Diego, CA) constructed by inserting a copy of the *R. eutropha phb*C gene into the chromosome. This strain does not produce PHAs from sugars or fatty acids because of the absence of enzymes for converting acetyl-CoA or fatty acid oxidation intermediates into the R-3-hydroxyacyl-CoA monomers. The *phaJ* gene encoding an enoyl-CoA hydratase (Fukui and Doi, *J. Bacteriol.* 179: 4821-30 (1997)), was isolated from chromosomal DNA prepared from *A. caviae* strain FA-440 (obtained from the Japanese Culture Collection under accession number FERM BP 3432 (U.S. Patent No. 5,292,860) by the polymerase chain reaction using the primers:

Ac3-5' (SEQ ID NO:15):

AGAATTCAGGAGGACGCCGCATGAGCGCACAATCCCTGG

and Ac3-3' (SEQ ID NO:16):

TTCCTGCAGCTCAAGGCAGCTTGACCACG

and a PCR reaction mixture obtained from Life Technologies (Gaithersburg, MD). The PCR program was 30 cycles of (95°C, 45s; 55°C, 45s; 72°C, 1 min.). Following PCR, the DNA fragment was digested to completion with EcoRI and PstI, gel purified and ligated into the EcoRI/PstI sites of plasmid pUC18Sfi (Herrero et. al.) to,obtain plasmid pMTXJ12. Transformants of *E. coli* MBX 240 containing plasmid pMTXJ12 were grown in Luria-Bertani medium containing 10 mM octanoate and 1mM oleate and ampicillin at 100

μg/ml. After growth at 37 °C for 48 hours, 50 ml of cells were harvested by centrifugation and lyophilized. Lyophilized cells were extracted with 8 ml chloroform for 16 hours and the PHA precipitated in a 10-fold excess of ethanol at 4 °C. The precipitated polymer was analyzed by gas chromatography and identified as PHBH copolymer containing 2.6% HH comonomer.

Example 5: Production of PHBH copolymers From Butanol in *E.coli* Expressing the *A. caviae* PHB polymerase and the *R. eutroph* Thiolase and Reductase Genes

PHBH was produced from glucose and butyrate in *E. coli* MBX1326 (identical to strain DC698, mel, *fad*R *ato*C (con) *adh*C81, *adh*R30 *ace*X, Clark & Rod, *J. Mol. Biol. Evol.* 25: 151(1987)) containing plasmids pMBXC12J12 and pSU18-AB1 as follows. The transformed cells were grown in 1L LB medium containing 5g/L butanol. Cells were harvested and analyzed as for Example 1. The genetically engineered cells produced a PHBH copolymer containing 1.2% HH.

Example 6: PHBH Synthesis in *E. coli* Using a Fatty Acid Biosynthesis Pathway

The fatty acid biosynthesis pathway is shown in Figure 5. R-3-hydroxyhexanoyl CoA also can be provided from intermediates from fatty acid biosynthesis. In *P. putida,* 3-hydroxyacyl CoA are provided from this pathway when this bacterium is grown on glucose or other carbohydrates. This pathway requires an activity that converts acyl ACP into acyl CoA, a reaction catalyzed by an ACP/CoA transacylase or by the combined action of an acyl ACP thioesterase and acyl CoA synthase. Introduction of this pathway in an *E. coli* strain that expresses the PHB biosynthetic genes and that has a constitutive fatty acid biosynthetic regulon (*fadR*$^+$), such as MBX689, results in the synthesis of R-3-hydroxyhexanoyl CoA.

Genes encoding the enzymes that facilitate the ACP to CoA transacylation are isolated in the following screen which employs the *lux* system from *V. fischeri*, as shown in Figure 10. Induction of the *lux* genes depends on the synthesis of autoinducer 3-ketohexanoyl homoserine lactone.

The precursors for this molecule are S-adenosylmethionine and 3-ketohexanoyl ACP. *E. coli* CGSC5638 has a mutation in the *fabD* gene encoding malonyl transacylase (Bouquin et al., *Mol. Gen. Genet.* 246: 628 (1995)) and is unable to synthesize acetoacetyl ACP. Hexanoate is provided to these cells for synthesis of long side chain fatty acids. In addition, a *fadR* mutation is introduced to degrade hexanoate to 3-ketohexanoyl CoA. In order for the cells to induce expression of the *lux* system, 3-kektohexanoyl CoA must be converted to 3-ketohexanoyl ACP. Gene libraries of various organisms then can be screened in this host, selecting positive clones for their ability to induce *lux* expression, which is identified as light emission due to the formation of inducer 3-ketohexanoyl homoserine lactone. Gene libraries are readily constructed from organisms of choice by isolating genomic DNA and cloning a representative collection of DNA fragments in plasmid vectors. Representative libraries should have 5,000 to 100,000 individual colonies. Libraries are made either as a broad-host-range library in vectors such as pLAFR3 or as *E. coli* libraries in vectors such as pUC19 or pBR322. Depending on the type of library and the method of introducing the library in the host of choice, the genomic DNA fragments are either large (17-30 kb) or relatively small (2-6 kb). Libraries are introduced into the screening strains by electroporation, transformation, or conjugation, depending on the host and the vector used.

We claim:

1. A method for the biological production of polyhydroxyalkanoates containing 3-hydroxyhexanoate comprising synthesizing the polyhydroxyalkanoate in a transgenic organism having at least one transgene encoding an enzyme selected from the group consisting of PHB polymerase, PHA polymerase, ß-ketothiolase, ß-ketoacyl-CoA reductase, D-specific enoyl-CoA hydratase, crotonase, butyryl-CoA dehydrogenase, and 3-hydroxybutyryl-CoA dehydrogenase integrated into the chromosome.

2. The method of claim 1 wherein the organism is a bacteria or plant.

3. The method of claim 2 wherein the organism is a plant selected from the group consisting of oil crop plants and starch accumulating plants.

4. The method of claim 3 wherein the plant is selected from the group consisting of Brassica, sunflower, soybean, corn, safflower, flax, palm, coconut, potato, tapioca, cassava, alfalfa, grass, and tobacco.

5. The method of claim 2 wherein the organism is a bacteria selected from the group consisting of *Escherichia, Klebsiella, Ralstonia, Alcaligenes, Pseudomonas,* and *Azotobacter.*

6. The method of claim 1 wherein the organism is genetically engineered to express or overexpress a PHA polymerase incorporating C_6 substrates.

7. The method of claim 6 wherein the enzyme is derived from *Aeromonas caviae, Comamonas testosteroni, Thiocapsia pfenigii, Chromatium vinosum, Bacillus cereus, Nocardia carolina, Nocardia salmonicolor, Rhodococcus ruber, Rhodcoccus rhodocrous,* and *Rhodospirilum rubrum.*

8. The method of claim 1 wherein the organisms are genetically engineered to redirect metabolites to production of 3-hydroxyhexanoyl-CoA.

9. The method of claim 8 wherein the organisms are genetically engineered using a D-specific enoyl-CoA hydratase gene.

10. The method of 9 wherein the hydratase gene is isolated from a bacteria selected from the group consisting of *R. eutropha, Klebsiella aerogenes, P. putida,* and *Aeromonas caviae.*

11. The method of claim 8 wherein the organisms are genetically engineered using a butyrate fermentation pathway.

12. The method of claim 11 wherein the butyrate fermentation pathway is from *Clostridium acetobutylicium* or *Thermoanaerobacterium thermosaccharolyticum.*

13. The method of claim 11 wherein the organisms are genetically engineered to convert butyrate to butyryl CoA or butyryl CoA to crotonyl CoA.

14. The method of claim 11 wherein the organisms are genetically engineered to express a broad range reductase that is active on C_6 substrates.

15. The method of claim 11 wherein the organisms are genetically engineered to express a polymerase that accepts 3-hydroxyhexanoyl CoA.

16. The method of claim 11 wherein the organisms are genetically engineered to express a thiolase accepting acetoacetyl CoA.

17. The method of claim 11 wherein the organisms are genetically engineered to express an enzyme selected from the group consisting of thiolases specific for 3-ketohexanoyl CoA, reductase active on 3-ketohexanoyl CoA, PHA polymerase that accepts 3-hydroxybutyryl CoA and 3-hydroxyhexanoyl CoA.

18. The method of claim 8 wherein the organisms are genetically engineered using fatty acid biosynthetic enzymes.

19. The method of claim 18 wherein the fatty acid biosynthetic enzymes are enzymes converting acyl ACP to acyl CoA.

20. The method of claim 19 where the enzymes are selected from the group consisting of ACP-CoA transacylase, acyl ACP thioesterase, and acyl CoA synthase.

21. The method of claim 20 wherein the enzymes are acyl ACP thioesterase and acyl CoA synthase.

22. The method of claim 18 wherein the enzymes are derived from *E. coli*.

23. The method of claim 8 wherein the organisms are genetically engineered using a fatty acid oxidation complex.

24. The method of claim 23 wherein the fatty acid oxidation complex comprises enzymes selected from the group consisting of enzymes epimerizing S-3 hydroxyhexanoyl CoA and enzymes reducing 3-ketohexanoyl CoA.

25. The method of claim 24 wherein the enzymes are derived from *Nocardia salmonicolor*.

26. The method of claim 24 wherein the enzymes for epimerization are derived from *Pseudomonas putida* FaoAB complex.

27. The method of claim 23 wherein the organism that is genetically engineered accumulates 3-ketohexanoyl CoA due to a lack of a thiolase.

28. A method for producing polyhydroxybutyrate-co-3-hydroxyhexanoate comprising feeding an organism

butyrate or butanol, and

another feedstock selected from the group consisting of glucose, sucrose, lactose, xylose, methanol, and combinations thereof.

29. A method for producing 3-hydroxyhexanoate copolymers comprising

identifying an organism capable of taking up butyrate and converting it to butyryl-CoA,

fermenting the organism in the presence of butyrate such that PHBH is produced, and

recovering the PHBH.

30. A method for producing 3-hydroxyhexanoate copolymers comprising

identifying bacteria capable of taking up butanol and converting it to butyryl-CoA,

fermenting the organism in the presence of butanol such that PHA is produced, and

recovering the PHA.

31. A genetically engineered organism for use in any of the methods of claims 1-30.

32. The organism of claim 31 wherein the organism is a bacteria.

33. The organism of claim 31 wherein the organism is a higher order plant.

34. A polyhydroxybutyrate-co-3-hydroxyhexanoate produced in a genetically engineered *Escherichia coli* K12.

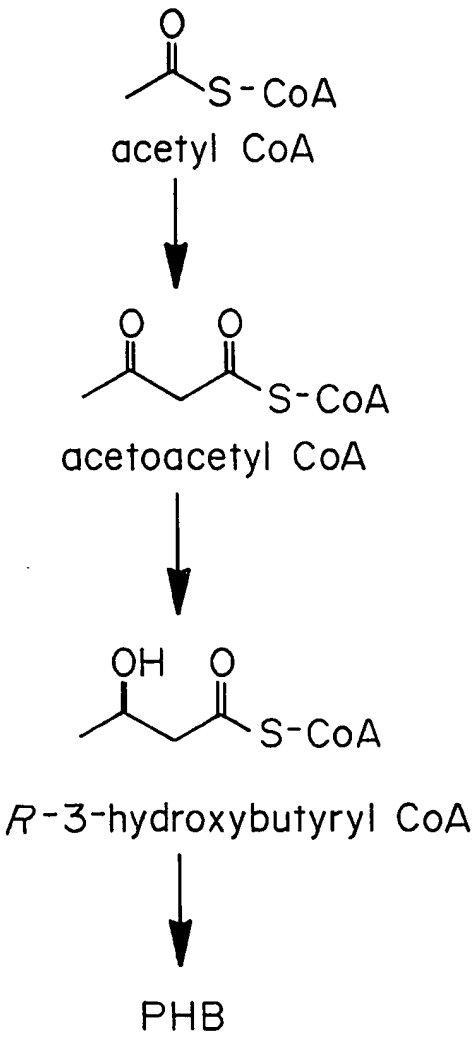

O
S-CoA
acetyl CoA
O
O
S-CoA
acetoacetyl CoA
OH
O
S-CoA
R-3-hydroxybutyryl CoA
PHB

FIG. 1

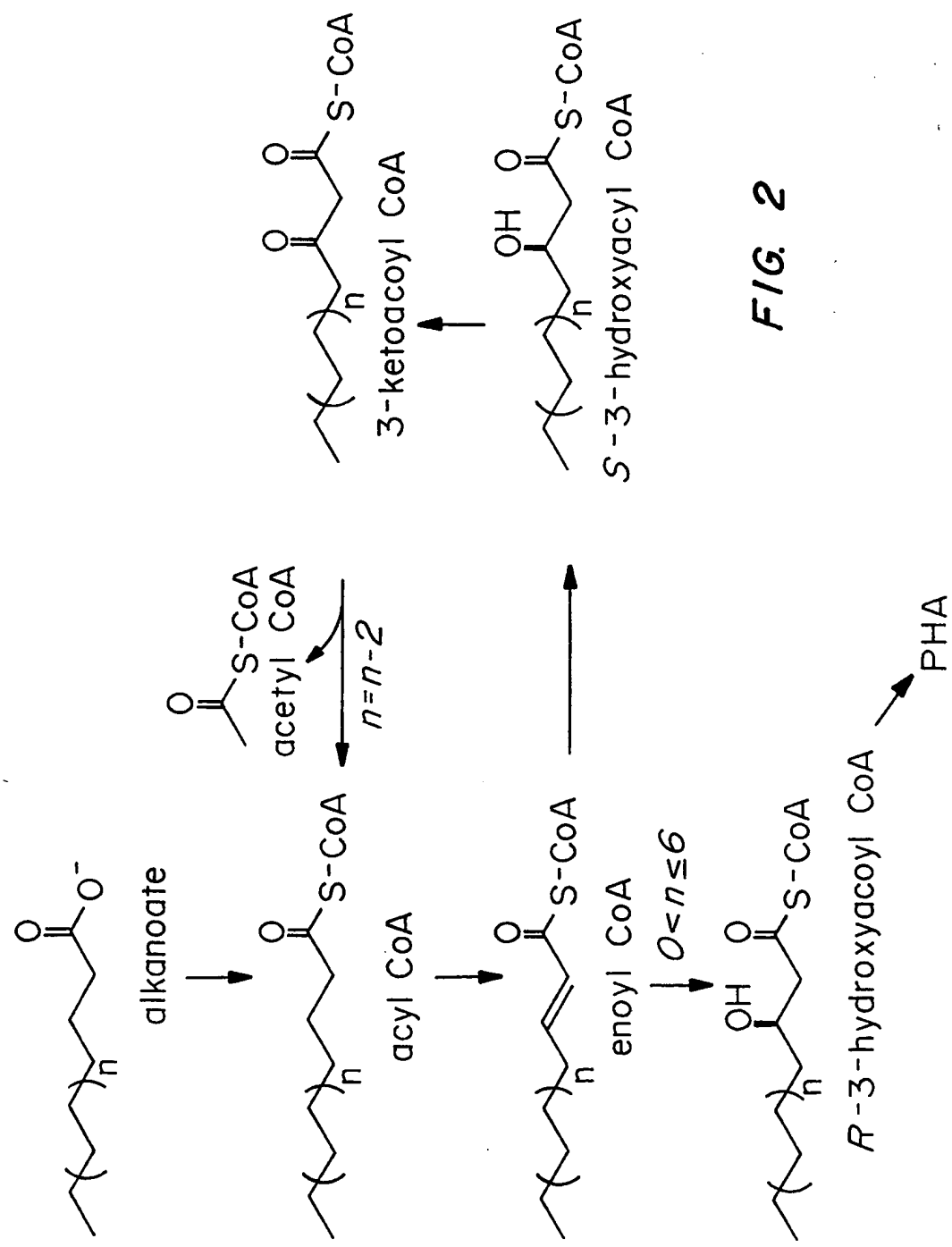
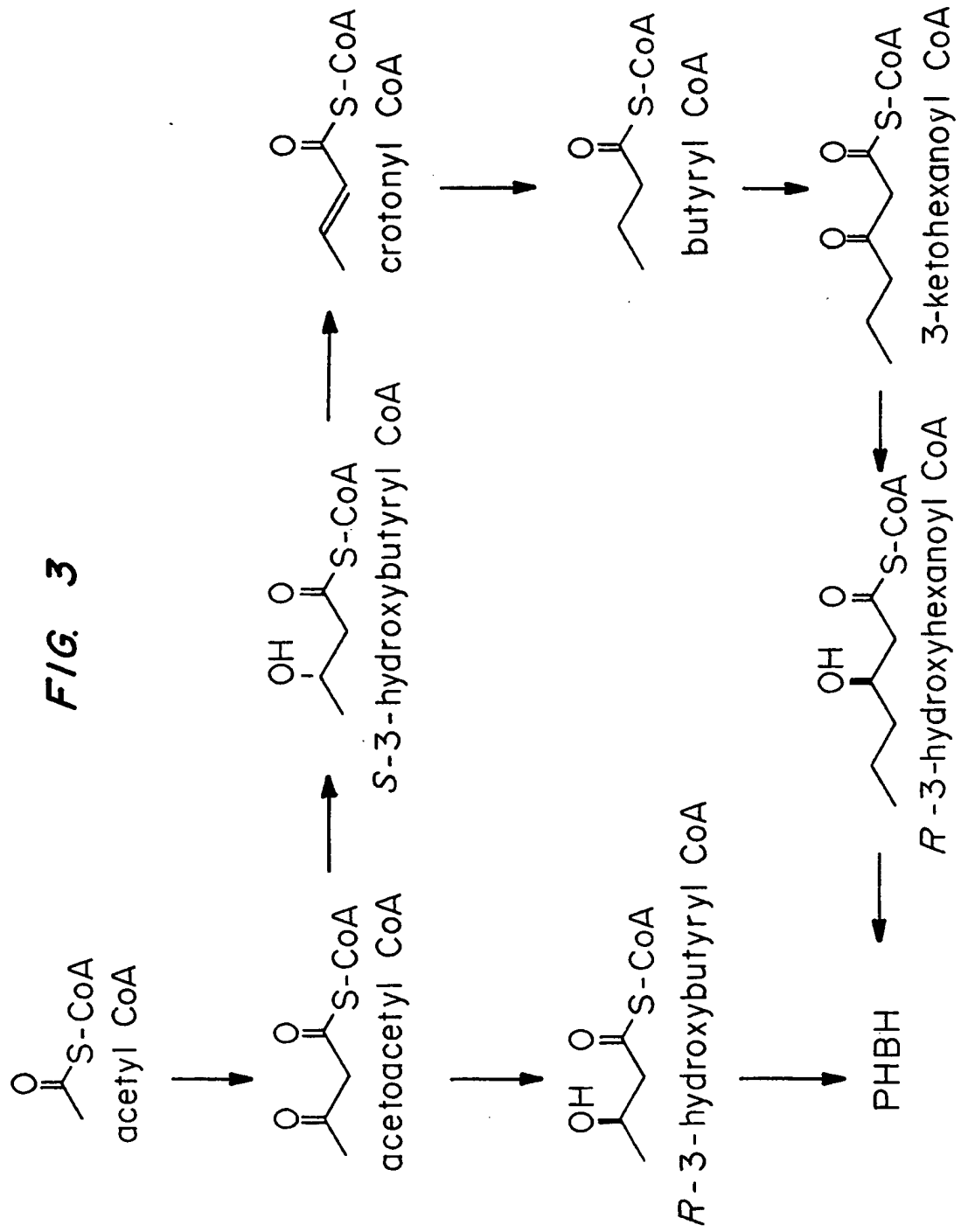

alkanoate
acyl CoA
enoyl CoA
O<n≤6
R-3-hydroxyacoyl CoA
PHA
acetyl CoA
n=n-2
3-ketoacoyl CoA
S-3-hydroxyacyl CoA
S-CoA

FIG. 2

FIG. 3


acetyl CoA
acetoacetyl CoA
S-3-hydroxybutyryl CoA
crotonyl CoA
butyryl CoA
3-ketohexanoyl CoA
R-3-hydroxybutyryl CoA
PHBH
R-3-hydroxyhexanoyl CoA
S-CoA
OH
O

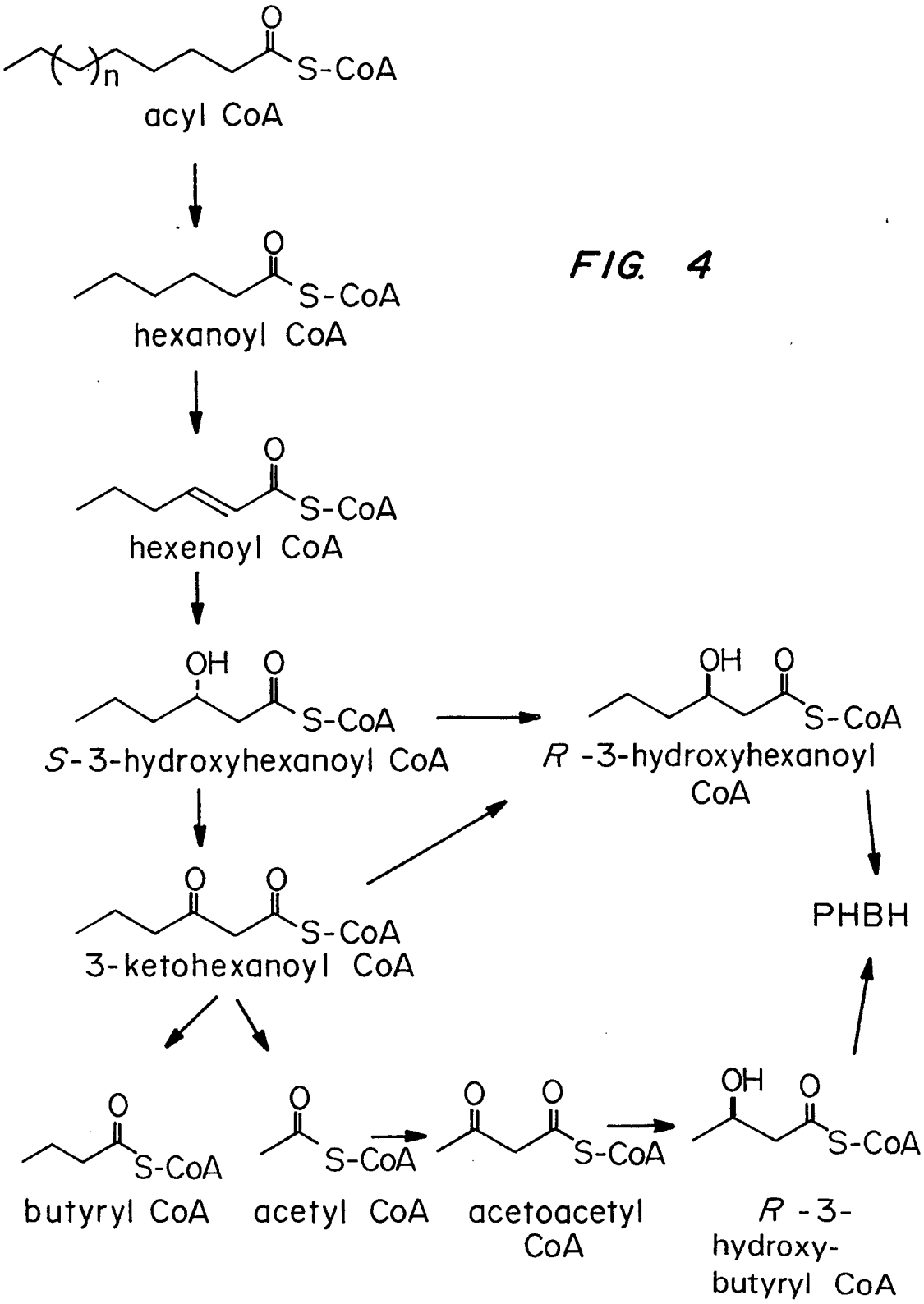

O
S-CoA
n
acyl CoA
hexanoyl CoA
hexenoyl CoA
OH
S-3-hydroxyhexanoyl CoA
R -3-hydroxyhexanoyl CoA
3-ketohexanoyl CoA
PHBH
butyryl CoA
acetyl CoA
acetoacetyl CoA
R -3-hydroxy-butyryl CoA

FIG. 4

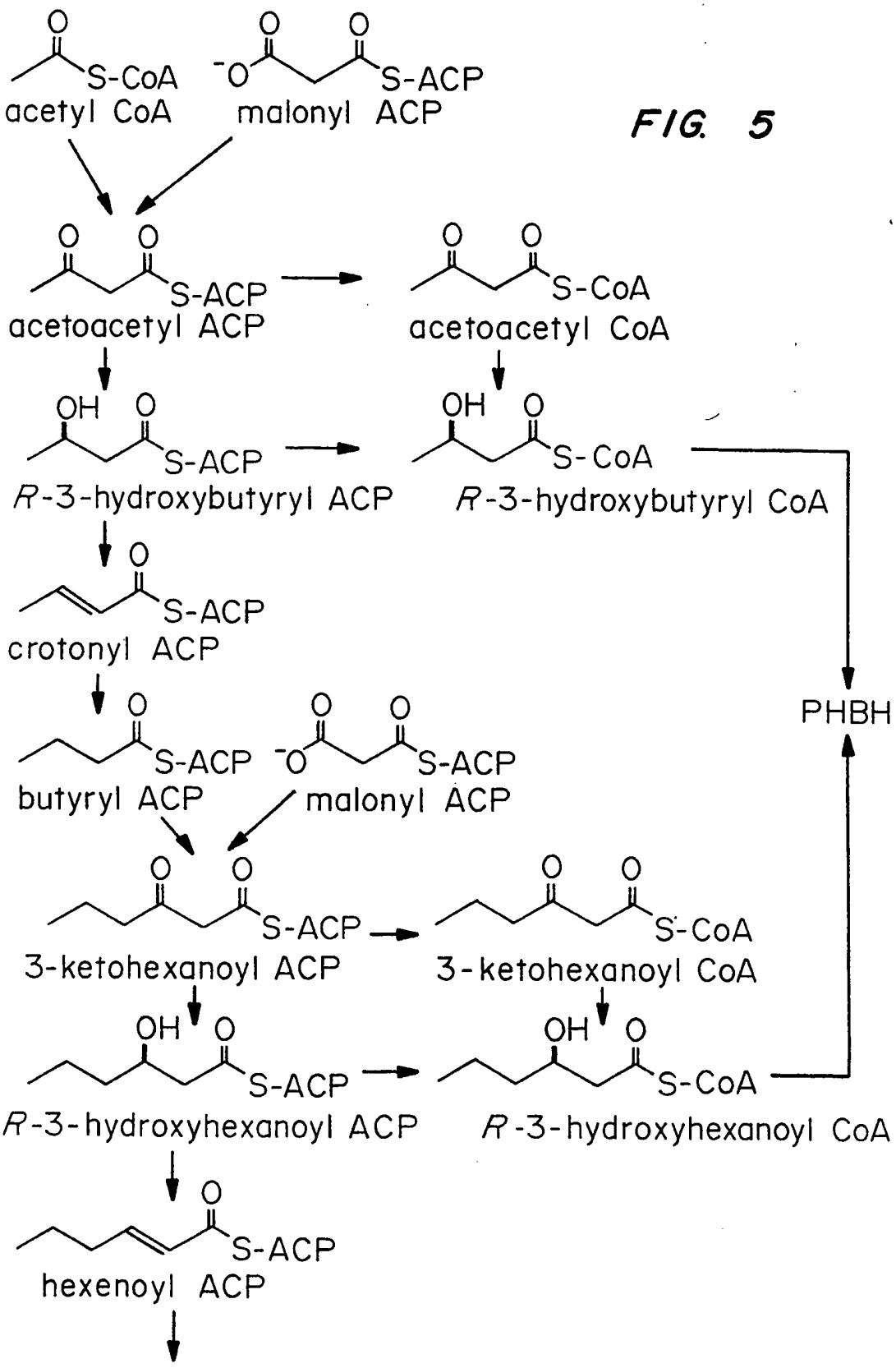
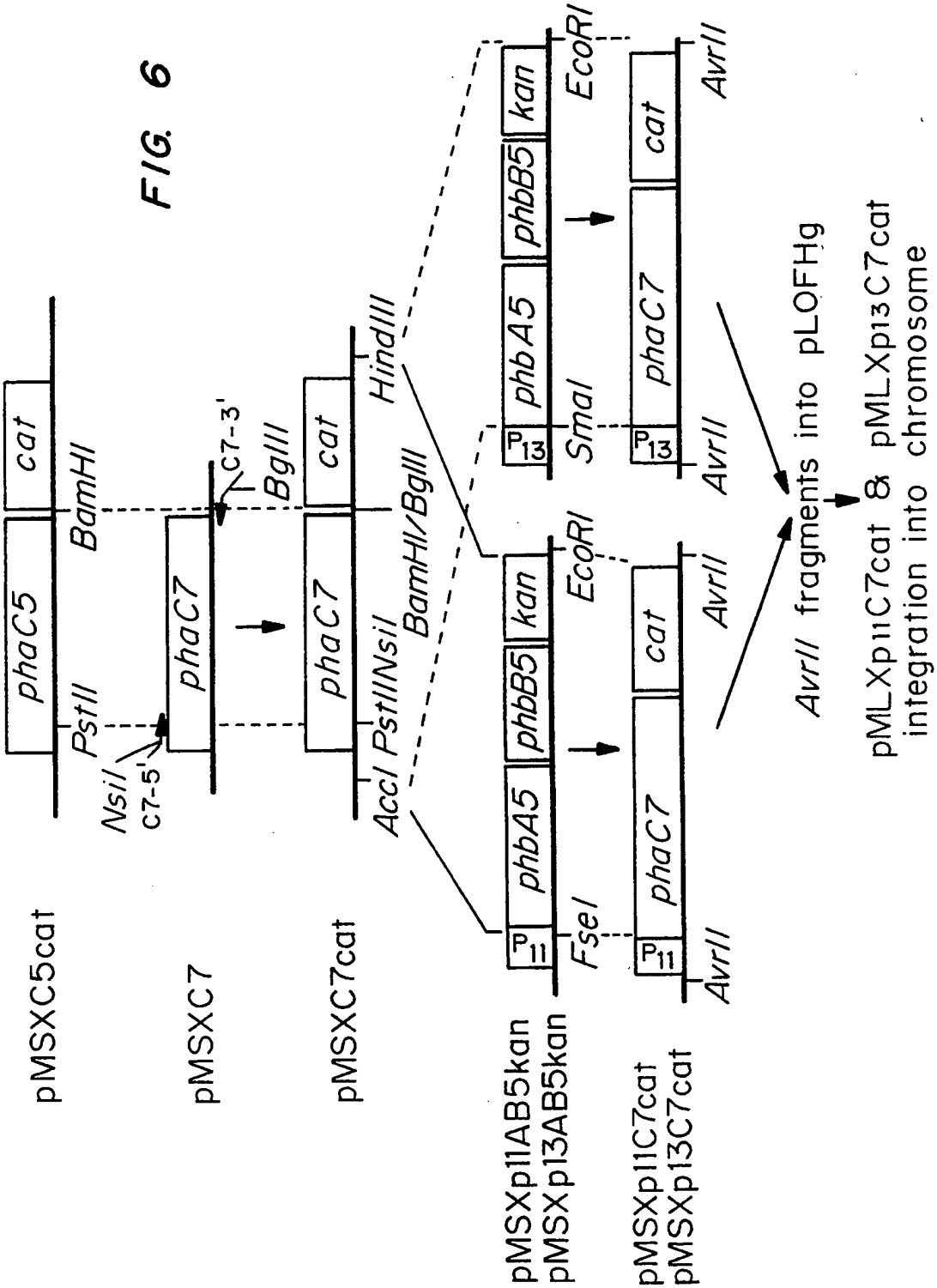

acetyl CoA
malonyl ACP
acetoacetyl ACP
acetoacetyl CoA
R-3-hydroxybutyryl ACP
R-3-hydroxybutyryl CoA
crotonyl ACP
butyryl ACP
malonyl ACP
PHBH
3-ketohexanoyl ACP
3-ketohexanoyl CoA
R-3-hydroxyhexanoyl ACP
R-3-hydroxyhexanoyl CoA
hexenoyl ACP

FIG. 5

FIG. 6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pMSXC7
pMSXC7cat
pMSXp11AB5kan
pMSXp13AB5kan
pMSXp11C7cat
pMSXp13C7cat
phaC5
cat
BamHI
PstII
NsiI
C7-5'
phaC7
C7-3'
BglII
HindIII
AccI PstI/NsiI
BamHI/BglII
phbA5
phbB5
kan
EcoRI
SmaI
AvrII
P11
P13
FseI
pMLXp11C7cat & pMLXp13C7cat integration into chromosome
AvrII fragments into pLOFHg

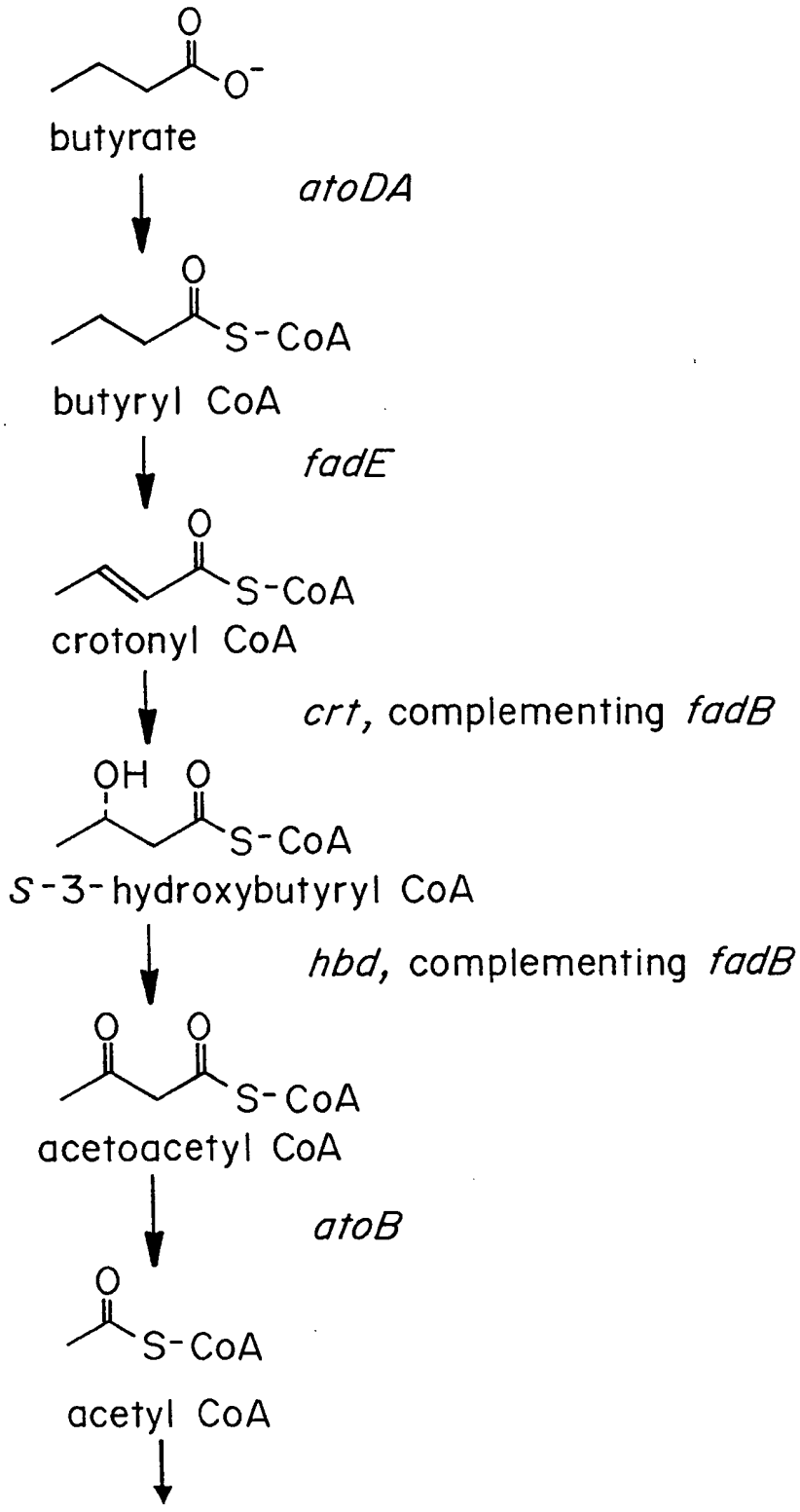
butyrate
atoDA
S-CoA
butyryl CoA
fadE
S-CoA
crotonyl CoA
crt, complementing fadB
OH
S-CoA
S-3-hydroxybutyryl CoA
hbd, complementing fadB
S-CoA
acetoacetyl CoA
atoB
S-CoA
acetyl CoA

FIG. 7

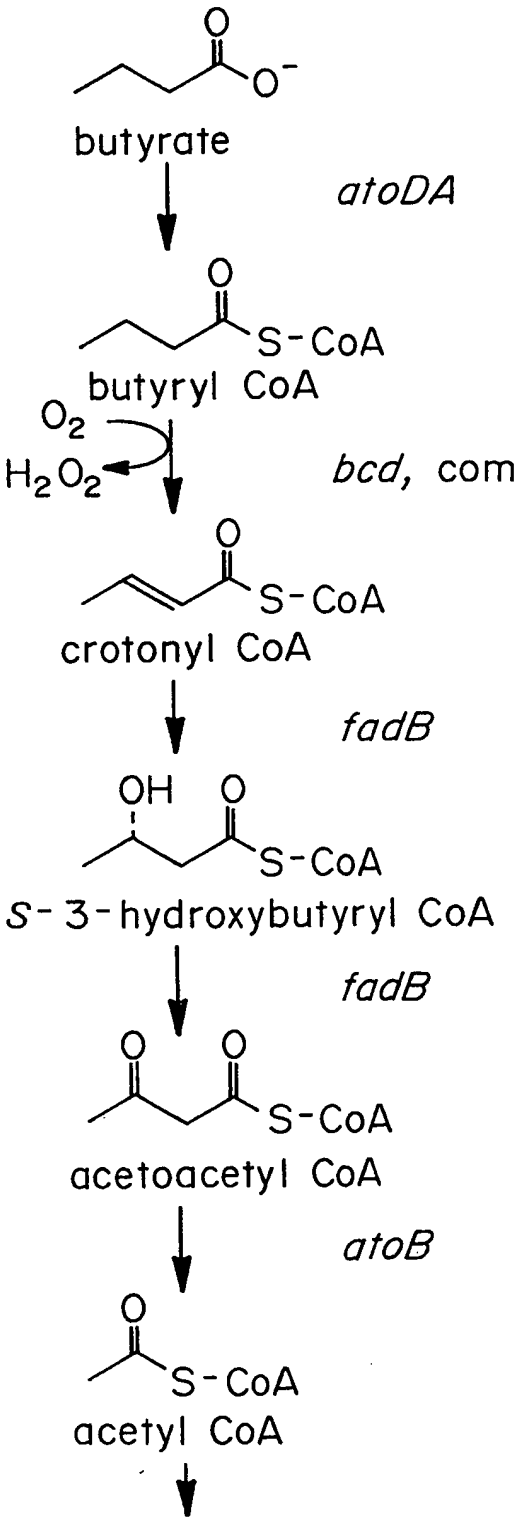
butyrate
atoDA
butyryl CoA
O_2
H_2O_2
bcd, complementing fadE
crotonyl CoA
fadB
S-3-hydroxybutyryl CoA
fadB
acetoacetyl CoA
atoB
acetyl CoA

FIG. 8

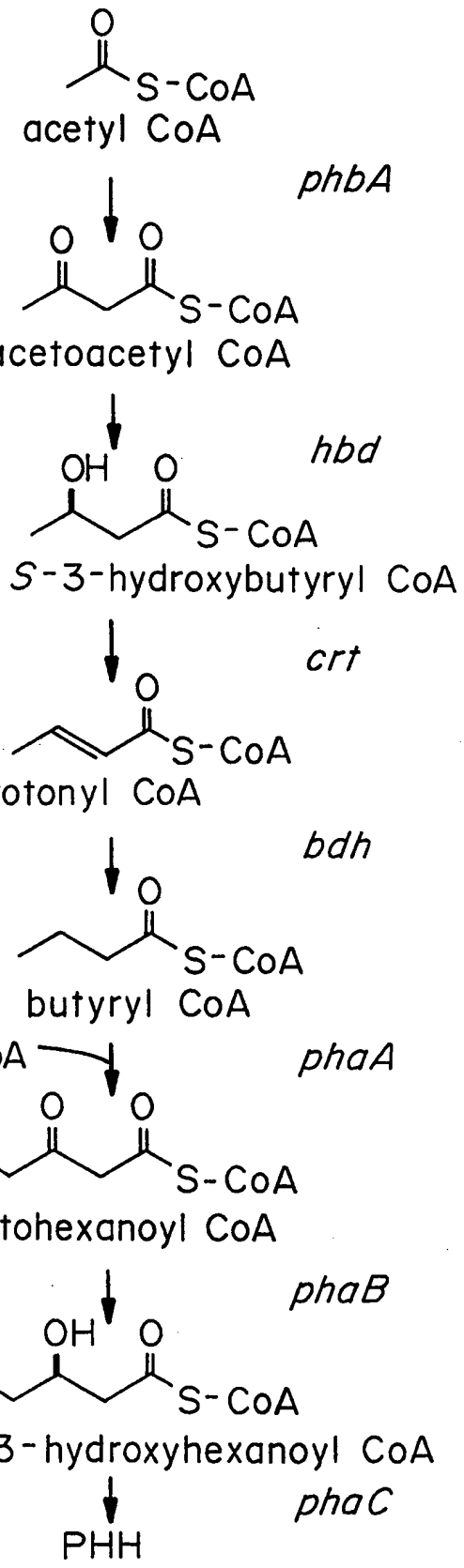
S-CoA
acetyl CoA
phbA
S-CoA
acetoacetyl CoA
OH
hbd
S-CoA
S-3-hydroxybutyryl CoA
crt
S-CoA
crotonyl CoA
bdh
S-CoA
butyryl CoA
acetyl CoA
phaA
S-CoA
3-ketohexanoyl CoA
phaB
OH
S-CoA
R-3-hydroxyhexanoyl CoA
phaC
PHH

FIG. 9

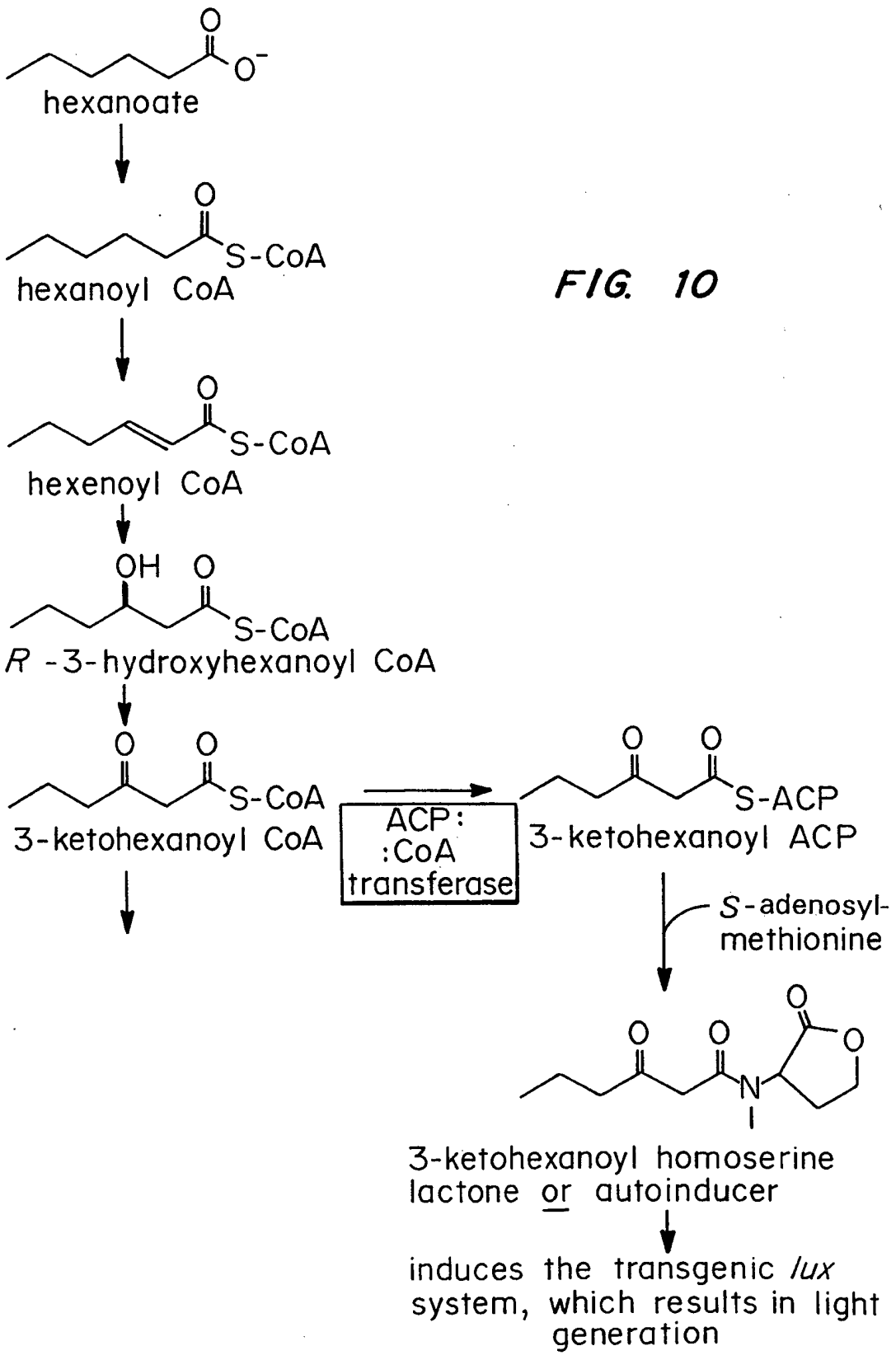
hexanoate
hexanoyl CoA
S-CoA
hexenoyl CoA
OH
R -3-hydroxyhexanoyl CoA
3-ketohexanoyl CoA
ACP: :CoA transferase
S-ACP
3-ketohexanoyl ACP
S-adenosyl-methionine
3-ketohexanoyl homoserine lactone or autoinducer
induces the transgenic lux system, which results in light generation

FIG. 10

SEQUENCE LISTING

<110> Metabolix, Inc.

<120> Transgenic Systems for the Manufacture of
Poly(3-Hydroxy-Butyrate-Co-3-Hydroxyhexanoate)

<130> MBX 020 PCT

<140> Not Yet Assigned
<141> 2000-01-21

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<151> 1999-01-22

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<170> PatentIn Ver. 2.1

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<210> 4
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oligonucleotide primer- C7-5'

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oligonucleotide primer- C7-3'

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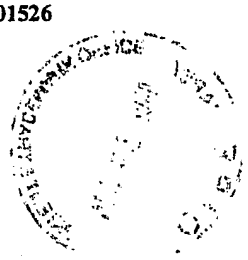

oligonucleotide primer- Ac3-5'

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<212> DNA
<213> Artificial Sequence

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oligonucleotide primer- Ac3-3'

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